

Conserved Herpesvirus Kinases Target the DNA Damage Response Pathway and TIP60 Histone Acetyltransferase to Promote Virus Replication

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SUMMARY

Herpesviruses, which are major human pathogens, establish life-long persistent infections. Although the α , β , and γ herpesviruses infect different tissues and cause distinct diseases, they each encode a conserved serine/threonine kinase that is critical for virus replication and spread. The extent of substrate conservation and the key common cell-signaling pathways targeted by these kinases are unknown. Using a human protein microarray high-throughput approach, we identify shared substrates of the conserved kinases from herpes simplex virus, human cytomegalovirus, Epstein-Barr virus (EBV), and Kaposi's sarcoma-associated herpesvirus. DNA damage response (DDR) proteins were statistically enriched, and the histone acetyltransferase TIP60, an upstream regulator of the DDR pathway, was required for efficient herpesvirus replication. During EBV replication, TIP60 activation by the BGLF4 kinase triggers EBV-induced DDR and also mediates induction of viral lytic gene expression. Identification of key cellular targets of the conserved herpesvirus kinases will facilitate the development of broadly effective antiviral strategies.

INTRODUCTION

As major human pathogens, herpesviruses establish life-long persistent infections that result in clinical manifestations ranging from mild cold sores to pneumonitis, birth defects, and cancers. Although the α , β , and γ herpesviruses infect different tissues and cause distinct diseases, they confront many of the same challenges in infecting their hosts, reprogramming cell gene expression, sensing and modifying cell-cycle state, and reacti-

vating the lytic life cycle to produce new virions and spread infection (Arvin et al., 2007). Whereas the α , β , and γ mammalian herpesviruses encode latency and transcriptional regulatory genes that are unique to each subfamily, lytic cycle genes, such as those encoding virion structural components and proteins involved in replication of the viral genomes, are more conserved across the order Herpesviridae. Among the conserved gene products are the orthologous serine/threonine protein kinases (UL13, UL97, BGLF4, and ORF36) encoded by herpes simplex type 1 (HSV1), human cytomegalovirus (HCMV), Epstein-Barr virus (EBV), and Kaposi's sarcoma-associated herpesvirus (KSHV), respectively (Gershburg and Pagano, 2008). These kinases are structurally similar to the cellular kinase cdk2 (Romaker et al., 2006) and are recognized to phosphorylate a number of cyclin-dependent kinase cellular targets, including pRb (Hume et al., 2008), condensin (Lee et al., 2007), stathmin (Chen et al., 2010), lamin A/C (Hamirally et al., 2009; Lee et al., 2008; Meng et al., 2010), elongation factor 1 delta (Kato et al., 2001; Kawaguchi and Kato, 2003; Kawaguchi et al., 2003), MCM4 (Kudoh et al., 2006), and p27/KIP1 (Iwahori et al., 2009), as well as viral targets, including KSHV bZIP (RAP) (Izumiya et al., 2007), EBV EBNA1 and virion proteins (Zhu et al., 2009), and HCMV UL69 (Rechter et al., 2009). Deletion of the protein kinases or inhibition of their activity has been shown to impair virus replication of HCMV and EBV in cultured cells (Gershburg et al., 2007; Prichard et al., 1999; Wolf et al., 2001) and to reduce the titer of HSV1 and murine γ herpesvirus 68 (γ -HV68) in infected mice (Shibaki et al., 2001; Tarakanova et al., 2007). Herpesvirus replication takes place against a background of cell-cycle arrest overlaid with a pseudo S phase environment, whereby virus replication becomes dissociated from cellular DNA replication but selectively utilizes machinery normally activated during S phase (Kudoh et al., 2005; Li and Hayward, 2011). The mimicry of cyclin-dependent kinase activity by the conserved herpesvirus protein kinases contributes to the creation of the pseudo S phase replication environment. This includes breakdown of the nuclear membrane, which is required for egress of virus capsids from the nucleus and is dependent in

infected cells on the viral protein kinase phosphorylation of lamin A/C (Hamirally et al., 2009; Lee et al., 2008; Meng et al., 2010).

Herpesvirus infection and lytic replication trigger the cellular DNA damage response. The induced DNA damage response is blunted during the establishment of latent herpesvirus infection, in EBV by the latency protein EBNA3C (Nikitin et al., 2010), and in HSV1 by the ICP0 protein (Lilley et al., 2010a). This attenuation of the response is necessary for effective establishment of viral latency. Conversely, aspects of the DNA damage pathway are selectively incorporated into the herpesvirus lytic replication program (Gaspar and Shenk, 2006; Kudoh et al., 2005; Lilley et al., 2005; Shin et al., 2006) and are necessary for efficient viral replication. In particular, early events such as activation of the DNA damage response kinase, ataxia telangiectasia mutated (ATM) protein, and phosphorylation of the ATM target H2AX are detected in cells undergoing lytic herpesvirus replication. The γ -HV68 protein kinase (orf36) and the EBV protein kinase BGLF4 have been shown to phosphorylate and activate ATM and H2AX (Tarakanova et al., 2007).

The nucleoside analog drugs acyclovir and ganciclovir, which are used to treat herpesvirus infections, require a monophosphorylation step that occurs in herpesvirus infected cells, but not in uninfected cells, and conserved protein kinases can mediate this phosphorylation (Gershburg et al., 2004; Meng et al., 2010; Moore et al., 2001; Sullivan et al., 1992). The multiple contributions of the conserved protein kinases to herpesvirus replication and spread also make these kinases potential antiviral drug targets, although to date, only one inhibitor of protein kinase enzymatic activity, maribavir, has entered clinical trials (Prichard, 2009).

The herpesvirus protein kinases have a broader substrate recognition than cellular cdk (Baek et al., 2002a; Cano-Monreal et al., 2008; Romaker et al., 2006; Zhu et al., 2009) and neither the full range of their substrates, nor the degree to which the substrates of individual conserved protein kinases overlap, is known. Comprehensive knowledge of common host targets would provide valuable insight into key host factors that facilitate herpesvirus replication and identify signaling pathways whose targeting in combination could enhance the effectiveness of antiviral treatments. Using a human protein microarray screen, we have identified more than 100 shared substrates of the α , β , and γ herpesvirus conserved kinases. Bioinformatic analyses of these shared substrates revealed a statistical enrichment of proteins involved in the DNA damage response. Follow-up experimentation highlighted the key contribution to herpesvirus replication of protein kinase-mediated-phosphorylation of the histone acetyltransferase TIP60, a regulator of the DNA damage response and of chromatin remodeling.

RESULTS

Common Host Substrate Identification for Conserved Herpesvirus Protein Kinases

To identify common substrates for the herpesvirus-conserved protein kinases, we performed assays on a human protein microarray composed of 4,191 unique human proteins, using the UL13, UL97, BGLF4, and ORF36 orthologous kinases encoded by the α , β , and γ viruses HSV1, HCMV, and EBV and KSHV, respectively (Figure 1A). Using normalized amounts of purified

viral kinases, as determined by autophosphorylation reactions, we identified 273, 178, 290, and 294 substrates of BGLF4, ORF36, UL97, and UL13, respectively, at a cutoff value of $SD \geq 3$ (Figure 1A and Figure S1 and Table S1 available online). Of the 643 nonredundant substrates collectively identified by the four kinases, 110 are shared by at least three kinases (Figure 1B). Gene ontology (GO) analysis of these 110 common substrates revealed involvement in eight major functional classes, whereas statistical analysis indicated that the DDR was significantly enriched ($p = 0.004$; hypergeometric test) (Figures 1B and 1C). In addition, proteins in this DDR category are also enriched for known association with viral infections ($p = 0.016$; Table S2).

An effective means for a virus to exploit the host is to target a master regulator that controls multiple downstream signaling pathways. To identify such a master regulator, we applied orthogonal analysis to the shared substrates by incorporating different types of data (e.g., protein-protein and enzyme-substrate interactions) and found a highly connected cluster of 12 proteins, all involved in the DDR (Figure 1C). Intriguingly, several proteins are either the direct targets (e.g., CHK1, RPA1, and RAD51) or downstream effectors of ATM kinase, which plays a crucial role in DDR (Harper and Elledge, 2007). ATM was not present on our protein microarrays. However, recent studies have shown that the activation of ATM's kinase activity in response to DNA damage is dependent upon TIP60 (Sun et al., 2005), one of the substrates that was common to the herpesvirus-conserved protein kinases (Figure 1C). Because TIP60 plays an important role in both DDR and transcription regulation through chromatin remodeling (Sapountzi et al., 2006; Squatrito et al., 2006), it is a candidate master regulator of the herpesvirus life cycle. Therefore, we focused on TIP60 and its role in herpesvirus replication.

EBV BGLF4 Regulates Lytic Replication through the Phosphorylation and Activation of TIP60

Choosing EBV as the primary model, we first tested whether TIP60 expression affected viral DNA replication. Both Akata (EBV⁺) B cells and SNU719 (EBV⁺) gastric carcinoma cells were transformed with individual shRNA lentiviral constructs to knock down TIP60 expression. As a surrogate for viral DNA replication, we measured the EBV genome copy following lytic induction of EBV by IgG crosslinking and bortezomib treatment, respectively. Knockdown of TIP60 was incomplete (Figure S2) but, nonetheless, reduced the number of EBV genomes by 60%–80% on both cell backgrounds and with both lytic induction treatments (Figure 2A). Measurement of extracellular infectious virus found an ~90% reduction upon TIP60 silencing (Figure 2B). Because the observed decrease was shown with two different shRNAs, the phenotype is unlikely to be due to off-target shRNA effects. These results indicate that TIP60 is a physiologically relevant substrate in the EBV life cycle.

To demonstrate that TIP60 is a target of the EBV kinase BGLF4 in cells, we first showed that TIP60 interacted with both a wild-type (WT) BGLF4 and a kinase-dead mutant (BGLF4^{KD}) in transfected cells using reciprocal coimmunoprecipitations (co-IPs) (Figures 3A and S3A). Note that, though the loss of BGLF4 kinase activity did not affect its interaction with TIP60, there was a change in TIP60 mobility with BGLF4^{KD}, indicating that BGLF4

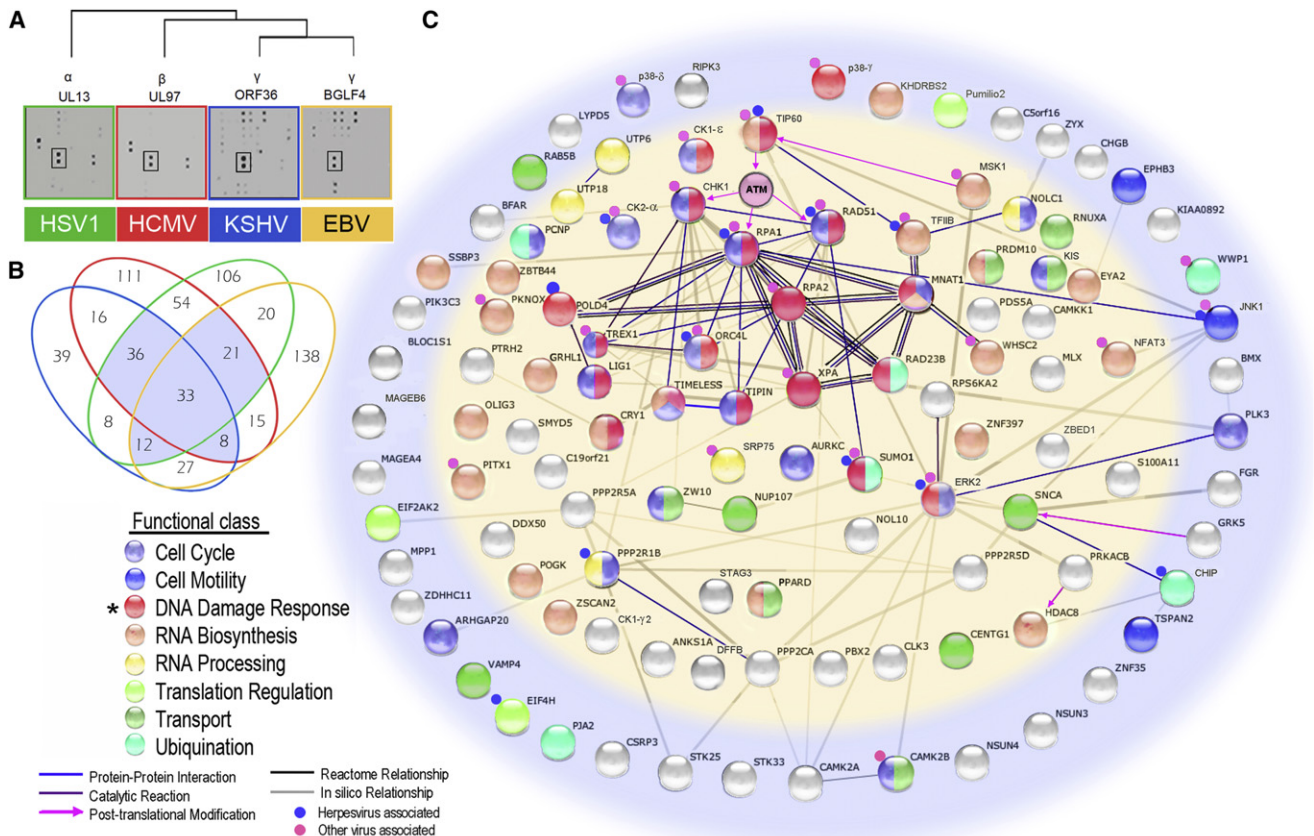


Figure 1. Identification of Common Host Protein Substrates for α , β , and γ Human Herpesvirus Protein Kinases: Enrichment of Proteins in the DNA Damage Pathway

(A) Autoradiograph showing representative sections of typical protein array phosphorylation assays performed using the four viral kinases. All substrates are printed in duplicate. The rectangle highlights a common substrate, Pumilio2.

(B) Venn diagram illustrating the overlap in substrate specificity of the herpesvirus protein kinases. Of 643 total substrates, the highlighted 110 host proteins were phosphorylated by at least three kinases. See also Table S1 and Figure S1.

(C) Interaction network for the 110 shared host proteins. Proteins are color coded by their functional classes. An asterisk indicates the enriched functional class of 19 proteins involved in DDR. Proteins in the inner oval (light yellow) are nuclear proteins, whereas the proteins in the outer ring are in other cellular compartments. Edges between the proteins represent known or predicted connections, such as protein-protein interactions, catalytic reactions, and enzyme-substrate relationships, obtained from the database STRING (<http://string-db.org/>). Note that ATM was not present on the human protein array. See also Tables S1 and S2.

plays a role in TIP60 phosphorylation. BGLF4-TIP60 interaction during EBV infection was validated using EBV-positive Akata (EBV⁺) cells induced into the lytic cycle by treatment with IgG to cross-link the B cell receptor and antibodies against endogenously expressed TIP60 (Figure 3B). Autologous EBV-negative Akata 4E3 cells (EBV⁻) served as a negative control. Having shown that BGLF4 directly phosphorylated TIP60 in vitro (Figure S3B), we sought to determine which sites on TIP60 were phosphorylated. In a previous study, phosphorylation at Ser86 and Ser90 of TIP60 was shown to enhance its HAT activity in vitro using histones as substrates. In addition, GSK3 β and CDK1/cyclin B were found to in vitro phosphorylate Ser86 and Ser90, respectively (Charvet et al., 2011; Lemerrier et al., 2003). Because BGLF4 and CDK1/cyclin B have overlapping substrate recognition (Hume et al., 2008; Zhu et al., 2009), we created TIP60 constructs carrying single or double mutations at Ser86 and Ser90. To show that BGLF4 directly phosphorylates TIP60 at Ser86, an in vitro phosphorylation assay was per-

formed. We found that the TIP60 pSer86-specific antibody detected phosphorylation of WT TIP60, but not phosphorylation of the S86A or S86/90A mutants (Figure 3C). Further, immunoblot analysis of TIP60 coprecipitated from WT TIP60-, S86A-, or S86/90A-transfected cell extracts by anti-BGLF4 antibody revealed that the S86A and S90A mutations each affected TIP60 mobility, with the effects of the double mutation being additive (Figure 3D). Phosphatase treatment increased the mobility of WT TIP60 coprecipitated with WT BGLF4 to equal that of WT TIP60 coprecipitated with BGLF4^{KD} and also equal to that of the S86/90A double mutant. This indicates that TIP60 Ser86/90 are major sites of phosphorylation by BGLF4. To further confirm Ser86 phosphorylation of TIP60 in vivo, we monitored Ser86 phosphorylation of endogenous TIP60 upon induction of WT BGLF4 or BGLF4^{KD} and found that Ser86 phosphorylation was dependent on the presence of BGLF4 kinase activity (Figure 3E, left). These results were further supported in lytically induced Akata (EBV⁺) cells, in which Ser86 phosphorylation of TIP60

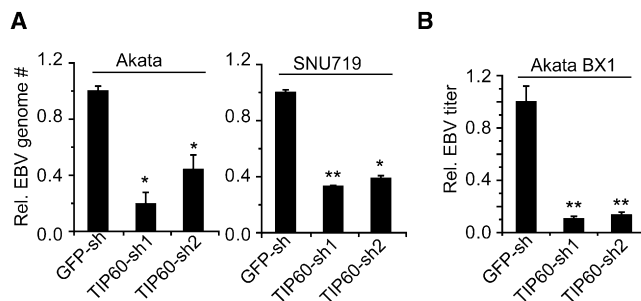


Figure 2. TIP60 Is Required for Efficient EBV Lytic Replication

(A) TIP60 silencing impairs lytic DNA replication. Relative viral genome copy numbers measured by qPCR in lytically induced Akata (EBV⁺) and SNU719 (EBV⁺) cells carrying control shRNA (GFP-sh) or TIP60 shRNAs (TIP60-sh1, TIP60-sh2). The experiments were performed in triplicate with \pm SD shown. * $p < 0.02$, ** $p < 0.01$. See also Figure S2A.

(B) TIP60 silencing reduces infectious virus production. Relative EBV titer produced by lytically induced Akata BX1 (EBV⁺) cells carrying control shRNA (GFP-sh) or TIP60 shRNAs (TIP60-sh1, TIP60-sh2) was measured using Raji cell infection assay. The experiments were carried out in triplicate with \pm SD shown. ** $p < 0.01$. See also Figure S2B.

was almost completely abolished upon BGLF4 knock down (Figure 3E, right).

To investigate whether the histone acetyltransferase (HAT) activity of TIP60 is affected by BGLF4 phosphorylation, we compared HAT activity of WT and S86/90A TIP60 coexpressed with either WT BGLF4 or BGLF4^{KD}. TIP60 was immunoprecipitated, and its HAT activity was measured in vitro (Figures 3F and S3C). TIP60s HAT activity in the presence of WT BGLF4 was 3-fold greater than that seen with BGLF4^{KD}, indicating that BGLF4's phosphorylation of TIP60 substantially enhances its HAT activity. This result was further supported by the observation that the S86/90A double mutation reduced TIP60's HAT activity to that of a HAT-deficient TIP60 mutant, regardless of the presence or absence of WT BGLF4 or BGLF4^{KD} (Figures 3F and S3C). Taken together, the data establish that BGLF4 interacts with and phosphorylates TIP60 to increase TIP60's HAT activity.

BGLF4 Induces the DNA Damage Response and Chromatin Remodeling through TIP60

TIP60 mediates chromatin remodeling, and TIP60 acetylation of ATM activates ATM autophosphorylation and ATM transphosphorylation of downstream targets such as those illustrated in Figure 4A. Although DDR and chromatin remodeling have been implicated in herpesvirus replication, the molecular mechanisms are poorly understood (Lilley et al., 2010b). Therefore, we examined whether BGLF4 regulates DDR and chromatin remodeling via TIP60. As shown in Figure 4B, the presence of a series of DNA damage markers, including pSer1981 of ATM, pThr68 of CHK2, and pSer139 of histone H2AX (γ -H2AX), is dependent on induction of WT BGLF4, but not BGLF4^{KD}, in Akata (EBV⁺) cells, and inhibition of ATM abolishes these effects. In addition, Lys5 acetylation of histone H2A (H2AK5^{Ac}), a known target of TIP60, is substantially enhanced upon BGLF4 induction regardless of ATM inhibition (Figure 4B, lanes 5 and 8). Moreover, in a time course of lytic induction in Akata (EBV⁺) cells, BGLF4 appearance coincides with TIP60 phosphorylation, ATM activa-

tion, and γ -H2AX generation (Figure S4A). Consistent with this result, when endogenous BGLF4 is knocked down after lytic induction, ATM Ser1981 autophosphorylation is reduced to the same level as that seen in TIP60 knockdown cells, suggesting that BGLF4-induced DDR depends on TIP60. Moreover, in the same context, the phosphorylation of ATM's downstream effectors, CHK2 (pThr68) and H2AX (pSer139 or γ -H2AX), and the acetylation of TIP60s direct target, H2AK5^{Ac}, are also BGLF4 dependent (Figure 4B, right).

To test whether BGLF4-TIP60-dependent activation of DDR via ATM plays a role in EBV lytic replication, we measured extracellular virus produced by Akata BX1 (EBV⁺) cells in the latent state (0 hr) or after lytic induction (48 and 96 hr) in the absence or presence of an ATM inhibitor. We found that EBV lytic replication was suppressed in a dose-dependent manner by ATM inhibition (Figures 4C and S4B), demonstrating the critical role of ATM in EBV lytic replication.

A recent study showed that inhibition of DDR kinases ATM and Chk2 markedly increases the efficiency of EBV latency establishment in B cells (Nikitin et al., 2010). Because TIP60 acts upstream of ATM and CHK2, we asked whether inhibition of TIP60 also increases EBV latency establishment. Using the GFP-tagged virus produced by Akata BX1 cells and a Raji B cell infection assay, we found that latency establishment was increased in Raji cells carrying TIP60 shRNA compared to cells carrying control shRNA (Figures 4D and S4C).

BGLF4 Induces the Expression of Key Lytic Viral Genes through TIP60

To further illustrate the integration of BGLF4 and DDR into EBV DNA replication, we demonstrated that BGLF4 was recruited to the EBV lytic replication origin (OriLyt) upon lytic induction and that its presence induced the recruitment of γ -H2AX and the accumulation of H2AK5^{Ac} at the same locus (Figure 4E). Because TIP60 is known to acetylate histones and regulate gene expression (Avvakumov and Côté, 2007; Baek et al., 2002b; Ikura et al., 2000), we reasoned that the accumulation of H2AK5^{Ac} at this promoter induced by TIP60 could also contribute to viral gene expression. Therefore, we investigated whether the OriLyt (*BHLF1*) promoter or other promoters are targeted by TIP60 during lytic induction.

We performed chromatin immunoprecipitation (ChIP) assays coupled with real-time PCR to quantitatively survey 18-well-annotated EBV promoter regions, including the OriLyt (*BHLF1*) promoter, for TIP60 occupancy. The selected promoters are distributed across the EBV genome (de Jesus et al., 2003) and control 22 EBV genes (Figure 5A). Using antibody against endogenous TIP60 in lytically induced Akata (EBV⁺) cells, we found that TIP60 associated with the *BHLF1* (OriLyt) and *RTA* promoters and also with both promoters (ED-L1 and L1-TR) that regulate *LMP1* (Figure 5A), whereas no significant enrichment of TIP60 was observed on the other tested promoters. These results indicate that TIP60 associates with specific EBV promoters. We next examined the dynamics of this relationship to compare TIP60 occupancy of the *BHLF1* (OriLyt), *RTA*, and *LMP1* promoters during latency and postlytic induction. TIP60 association was not detected during latent infection of Akata (EBV⁺) cells, but TIP60 was recruited to all three promoters at 12 hr postinduction and remained associated at 24 hr (Figure 5B, top). In contrast, the

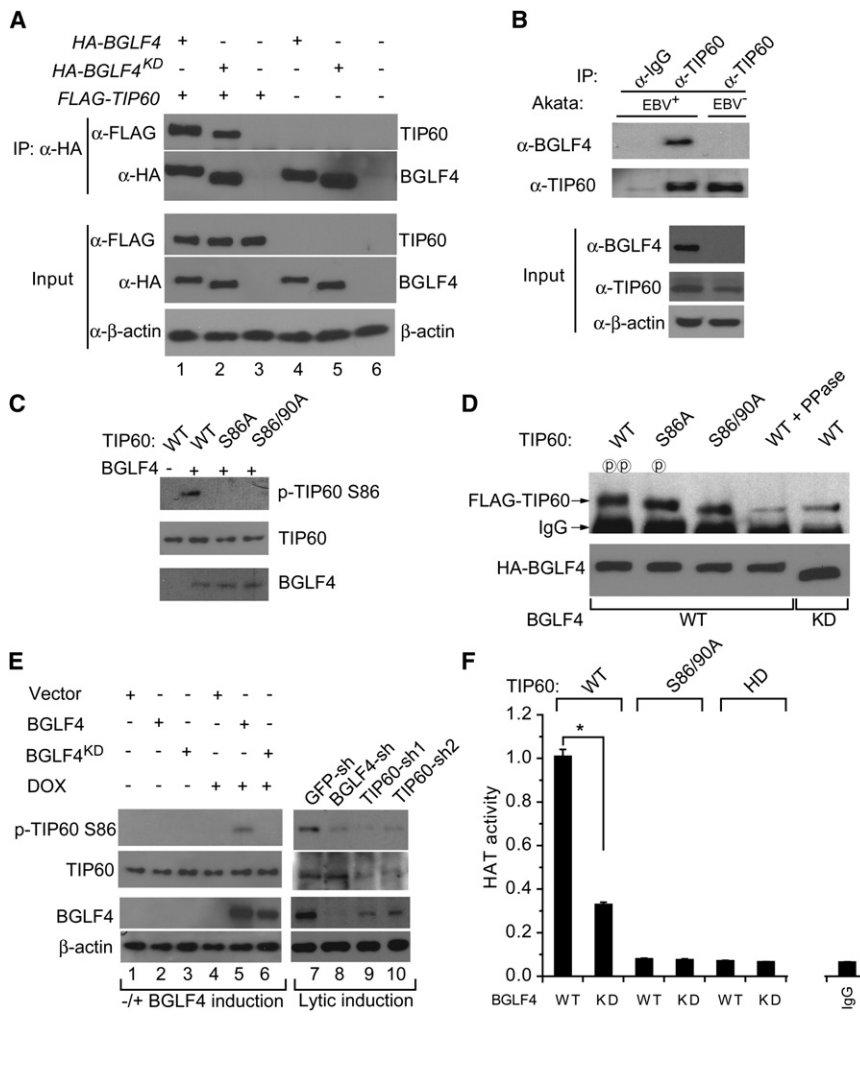


Figure 3. BGLF4 Interacts with, Phosphorylates, and Activates TIP60

(A) Both EBV BGLF4 and kinase-dead BGLF4 interact with TIP60. Western blot analysis of transfected 293T cell extracts showing coprecipitation of TIP60 with BGLF4. BGLF4^{KD}, BGLF4 kinase-dead mutant. Input, 2% whole-cell lysate used for IP. See also Figure S3A.

(B) Interaction between endogenous TIP60 and EBV BGLF4. Lytically induced Akata (EBV⁺) and Akata 4E3 (EBV⁻) cell extracts were immunoprecipitated with control IgG or anti-TIP60 antibodies, and the precipitated proteins were immunoblotted with the indicated antibodies. Input, 1% whole-cell lysate used for IP.

(C) BGLF4 phosphorylates TIP60 at S86 in vitro. Western blot analysis after in vitro phosphorylation reactions with indicated combinations of BGLF4 and wild-type or mutant TIP60.

(D) TIP60 Ser86 (S86) and Ser90 (S90) are substrates for BGLF4. Immunoblot comparing the mobility of BGLF4- and kinase-dead BGLF4-coprecipitated wild-type and mutant FLAG-TIP60 with and without phosphatase treatment. 293T cells were transfected as indicated and then treated with 20 μ M roscovitine for 12 hr before harvest. See also Figure S3B.

(E) BGLF4 induces TIP60 S86 phosphorylation in vivo. Western blot analysis of cell extracts from Akata (EBV⁺) cells carrying empty vector, BGLF4, or BGLF4^{KD}, with or without doxycycline (DOX, 20 ng/ml) treatment, and cell extracts from lytically induced Akata (EBV⁺) cells carrying control GFP, BGLF4, or TIP60 shRNAs.

(F) BGLF4 increases TIP60 HAT activity. Relative HAT activity of wild-type TIP60 (WT), phosphorylation-deficient TIP60 (S86/90A), and HAT-dead TIP60 (HD) immunoprecipitated from 293T cells transfected with wild-type BGLF4 (WT) or BGLF4^{KD} (KD) constructs. The experiments were carried out in triplicate with \pm SD shown. * $p < 0.01$. Immunoprecipitated TIP60 loading controls are shown in Figure S3C.

BMRF1 lytic promoter was not occupied by TIP60 during the course of lytic induction. To determine BGLF4's role in this process, we used shRNA lentiviral constructs to knock down BGLF4 expression in Akata (EBV⁺) B cells and then examined TIP60's recruitment to the *BHLF1*, *RTA*, and *LMP1* promoters during the course of EBV lytic induction (Figure 5B, middle). Quantitative measurement by qPCR showed that TIP60's occupancy on the three promoters was reduced by at least 50% between 12 and 24 hr postinduction (Figure S5A). Thus, BGLF4 enhances TIP60's recruitment to these three viral promoters.

Importantly, the three EBV genes targeted by TIP60 play key roles in viral replication. RTA is one of two key transcriptional activators that drive early and late lytic EBV gene expression (Zalini et al., 1996). The *BHLF1* (*OriLyt*) promoter is an essential component of the viral lytic origin of replication (Schepers et al., 1993). *LMP1* is a latency gene, but its expression is upregulated in the lytic cycle, where LMP1 provides key functions for cell survival and virus release (Ahsan et al., 2005; Dirmeier et al., 2005; Uchida et al., 1999). To correlate TIP60 recruitment and BGLF4 function with the efficiency of expression of these EBV

genes, we generated Akata (EBV⁺) cells that expressed BGLF4 shRNA (BGLF4-sh), TIP60 shRNA (TIP60-sh), or control GFP shRNA (GFP-sh). In the control GFP-sh Akata cells, as expected, these three genes and *BMRF1* were highly upregulated at 12 and 24 hr postinduction (Figure 5B, bottom). However, in BGLF4-sh and TIP60-sh cells (Figures 5B, bottom, and S5B), the expression level of *BHLF1*, *RTA*, and *LMP1* was significantly reduced at both time points, whereas *BMRF1* expression was minimally affected. *TIP60* knockdown had a greater negative impact than *BGLF4* knock down (Figure 5B, bottom). To summarize, the results reveal that EBV exploits TIP60 via BGLF4 phosphorylation to drive lytic viral gene expression. RTA-induced transcription of BGLF4 leads to reinforced RTA transcription and, consequently, to enhanced expression of the RTA-regulated lytic viral replication program (Wang et al., 2010).

Conserved Role for TIP60 in Herpesvirus Replication

Finally, we tested whether the interplay between the viral kinases and TIP60 is conserved in the other herpesviruses. Using the

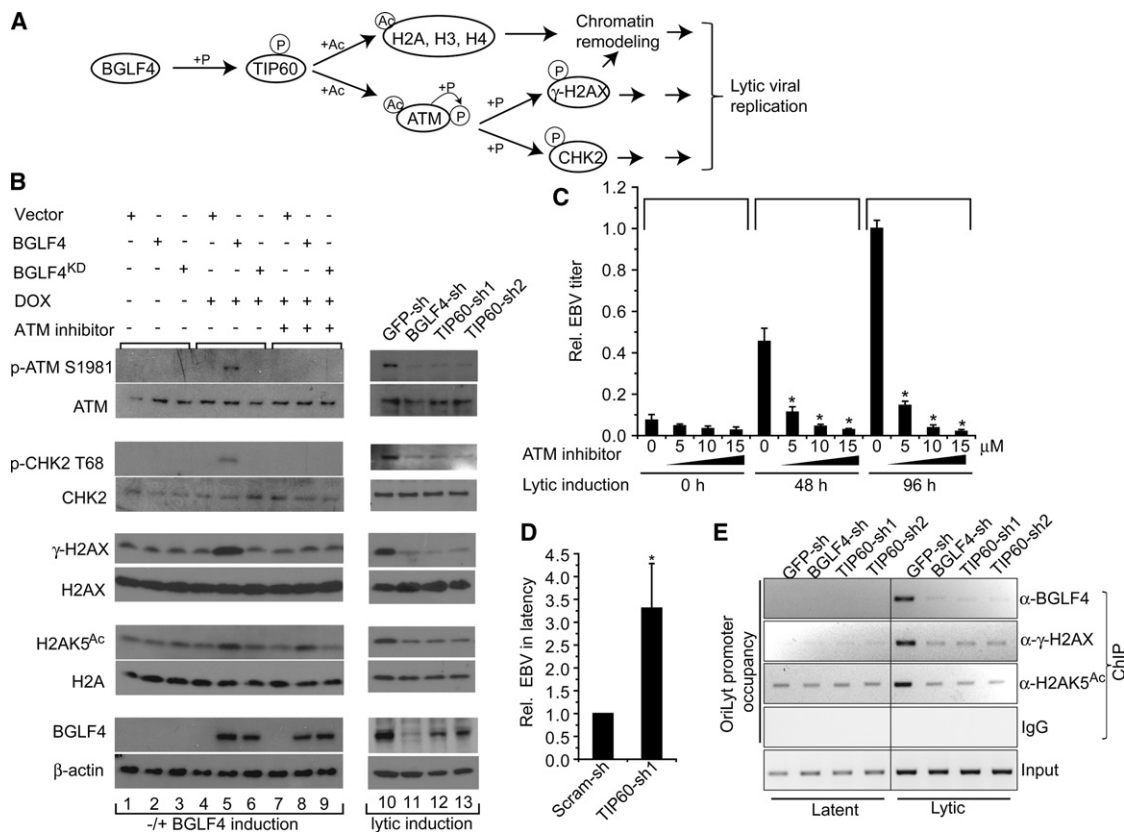


Figure 4. BGLF4 Induces the DNA Damage Response through TIP60

(A) Schematic illustration of BGLF4's potential function in DDR and chromatin remodeling through TIP60 phosphorylation. P, phosphorylation; Ac, acetylation. (B) BGLF4 induces histone acetylation and ATM activation via TIP60 phosphorylation. Western blot analysis of cell extracts from Akata (EBV⁺) cells carrying empty vector, BGLF4, or BGLF4^{KD}, with or without doxycycline (DOX, 20 ng/ml) or ATM inhibitor (KU55933, 15 μM) treatment, as indicated, and cell extracts from lytically induced Akata (EBV⁺) cells carrying control GFP, BGLF4, or TIP60 shRNAs. See also Figure S4A. The data are representative of at least two independent biological replicates.

(C) ATM inhibitor inhibits EBV lytic replication. Relative EBV titer of lytically induced Akata BX1 (EBV⁺) cells in the absence or presence of ATM inhibitor (KU55933), as indicated, was measured using Raji cell infection assay. The experiments were carried out in triplicate with ± SD shown. *p < 0.01. See also Figure S4B.

(D) TIP60 knockdown increases the efficiency of EBV latency establishment. EBV BX1 virus was used to infect Raji cells carrying control scramble shRNA (Scram-sh) or TIP60 shRNA (TIP60-sh1). Phorbol-12-myristate-13-acetate (TPA) (20 ng/ml) and sodium butyrate (3 mM) were added 6 days postinfection, and the number of the GFP-positive Raji cells was calculated to determine the efficiency of latency establishment. *p < 0.01. See also Figure S4C.

(E) Lytic induction results in the recruitment of BGLF4 and γ-H2AX to the EBV lytic replication origin (OriLyt) promoter and enrichment of histone acetylation (H2A Lys5 acetylation, H2AK5^{Ac}) on this promoter. ChIP-PCR analysis performed on Akata (EBV⁺) cells carrying indicated shRNAs showing BGLF4, γ-H2AX, and H2AK5^{Ac} enrichment at the OriLyt promoter after lytic induction.

same approaches described above, we showed that KSHV ORF36, HCMV UL97, and, to a lesser extent, HSV1 UL13 phosphorylated and increased the mobility of TIP60 in cotransfected HeLa cells (Figure 6A) and interacted with TIP60 in transfected 293T cells (Figures 6B, 6C, S6A, and S6B). In addition, we tested for recruitment of TIP60 at the HCMV lytic replication origin (OriLyt) and found that, similar to EBV, TIP60 was recruited to HCMV oriLyt at 24, 48, and 96 hr postinfection (hpi) (Figure 6D). Furthermore, knockdown of TIP60 in HCMV-infected cells significantly reduced production of extracellular HCMV virion DNA (Figures 6E and S6C). HCMV lytic replication was also significantly suppressed by an ATM inhibitor in a dose-dependent manner (Figures 6F and S6D), suggesting that the mechanism of inhibition parallels that shown for EBV. These results demonstrate that the viral kinase-TIP60 partnership is conserved and represents a common virus-host interaction.

DISCUSSION

High-throughput technology is emerging as a powerful tool for the discovery of factors involved in pathogen-host interactions (Brass et al., 2009; Calderwood et al., 2007; Karlas et al., 2010; König et al., 2010; Shapira et al., 2009). Here, we took a protein microarray approach to identify enzyme-substrate interactions for four conserved human herpesvirus kinases, with the hypothesis that the common substrates would reveal host pathways that are critical for replication across the herpesvirus family. By analyzing more than 100 shared host substrates, we identified the DDR pathway as a central target of the conserved herpesvirus kinases. Mechanistic studies showed that, in the absence of external DNA damage cues, the EBV kinase phosphorylated and activated the histone acetyltransferase TIP60, an upstream master regulator of DDR. In addition,

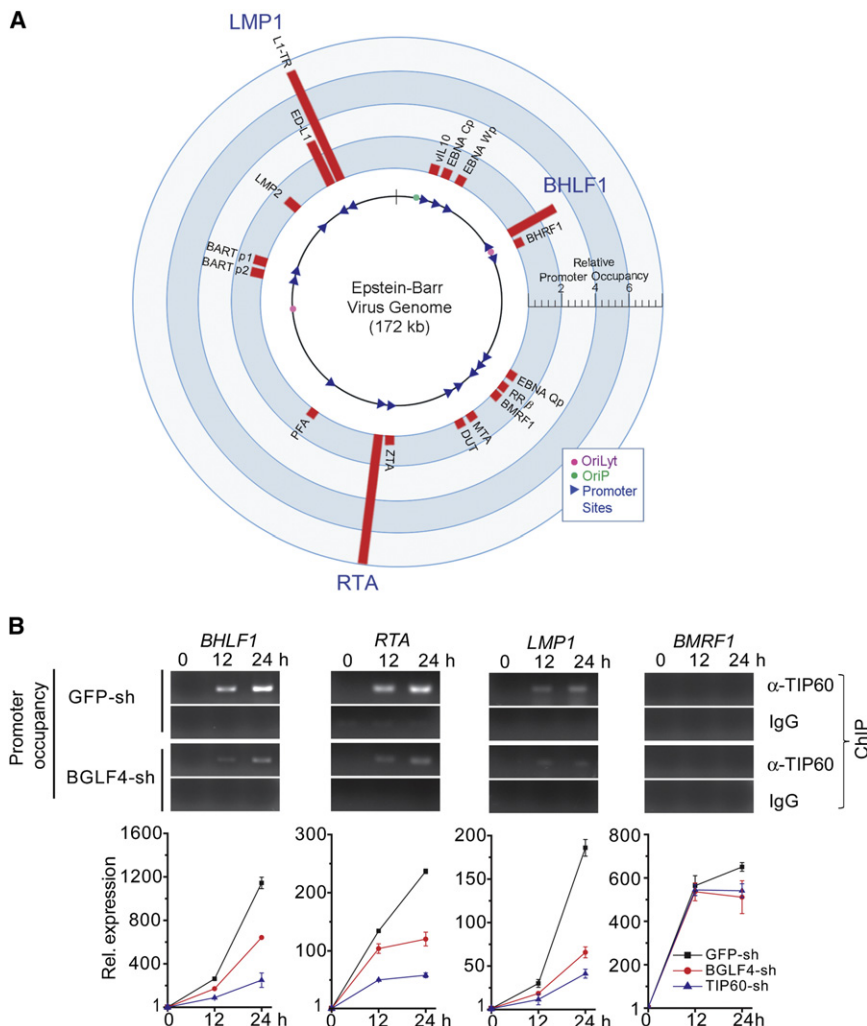


Figure 5. Regulation of EBV Lytic Gene Expression through TIP60

(A) TIP60 recruitment to EBV promoters. The EBV genome annotated with the 18 tested promoters (triangles) and origins of DNA replication (dots). (Red bars) Relative TIP60 occupancy normalized to the IgG control.

(B) Impact of TIP60 recruitment and BGLF4 activity on EBV lytic gene expression. (Top) ChIP-PCR analysis performed on Akata (EBV⁺) cells showing TIP60 enrichment at the *BHLF1*, *RTA*, and *LMP1* (*L1-TR*) promoters after lytic induction, but not the *BMRF1* lytic promoter. (Middle) Recruitment of TIP60 to the *BHLF1*, *RTA*, and *LMP1* (*L1-TR*) promoters was reduced in BGLF4-sh Akata (EBV⁺) cells. (Bottom) RT-qPCR analysis of mRNA levels for the corresponding genes and a nonenriched gene (*BMRF1*) in Akata (EBV⁺) cells carrying the indicated shRNAs. The experiments were carried out in triplicate with \pm SD shown. See also Figure S5.

We find here that TIP60 inhibition with shRNA also increases latency establishment, implying that TIP60 is an upstream mediator of DDR induced upon EBV infection. Interestingly, BGLF4 is present in the EBV tegument (Asai et al., 2006) and, consequently, is introduced into cells upon EBV infection. Therefore, BGLF4 would be available to initiate a transient activation of TIP60, and the DDR and BGLF4/TIP60 partnership may be an important factor in inducing a cellular environment that is hostile to latency establishment.

In counterpoint, we demonstrate that TIP60 plays a positive role in the lytic

TIP60 was integrated into the virus lytic program by recruitment to the viral chromatin, where TIP60 activated specific EBV genes critical for viral replication.

TIP60 was originally identified as a partner of the HIV type 1 (HIV-1) transactivator, Tat (Kamine et al., 1996), and is targeted by several viruses. Human T cell lymphotropic virus type 1 (HTLV-1) p30^{II} enhances Myc transforming activity through stabilizing Myc-TIP60 transcriptional interactions (Awasthi et al., 2005). TIP60 interaction with viral TAT, E6, and UL27 proteins encoded by HIV-1, human papillomavirus (HPV), and HCMV, respectively, induces TIP60 degradation (Col et al., 2005; Jha et al., 2010; Reitsma et al., 2011), which is believed to enable establishment of viral latency and enhance virus-induced oncogenesis. In the case of HCMV, viruses deleted or mutated for the UL97 protein kinase escape through secondary mutations in the UL27 protein that degrades TIP60 (Chou, 2009; Reitsma et al., 2011). A recent study by Nikitin et al. found that the DDR induced upon EBV infection is a robust host antiviral defense, and EBV employs countermeasures to overcome the growth inhibitory effects of the host DDR in order to establish latency (Nikitin et al., 2010). These authors found that treatment of B cells with an ATM inhibitor increased latency establishment.

replication of herpesviruses: TIP60 shRNA significantly reduces virus production from β and γ herpesvirus-infected cells. In the case of EBV, TIP60 HAT activity is enhanced via phosphorylation by the EBV-encoded protein kinase BGLF4 at the same sites that are phosphorylated by CDC2/CDK1 and GSK3 β (Charvet et al., 2011; Lemerrier et al., 2003). This interaction is sufficient to trigger DDR. DDR plays an important role in the lytic viral life cycle. EBV lytic replication elicits DDR by triggering ATM autophosphorylation and activation. Activated ATM phosphorylates its downstream targets, such as H2AX, p53, CHK2, and RPA2, and phosphorylated ATM, RPA2, and Mre11/Rad50/Nbs1 (MRN) complexes are recruited to replication compartments in nuclei during EBV lytic replication (Kudoh et al., 2005; Kudoh et al., 2009). However, the mechanism of virus-triggered ATM activation has been elusive. Although γ -HV68 kinase orf36 and EBV BGLF4 have been found to directly phosphorylate H2AX, this phosphorylation was reduced significantly in ATM-deficient cells (Tarakanova et al., 2007) and also, as shown here in Figure 4B, in cells treated with an ATM inhibitor. As summarized in Figure 7, our experiments mechanistically link the viral kinases to ATM and its downstream targets CHK2 and H2AX via TIP60.

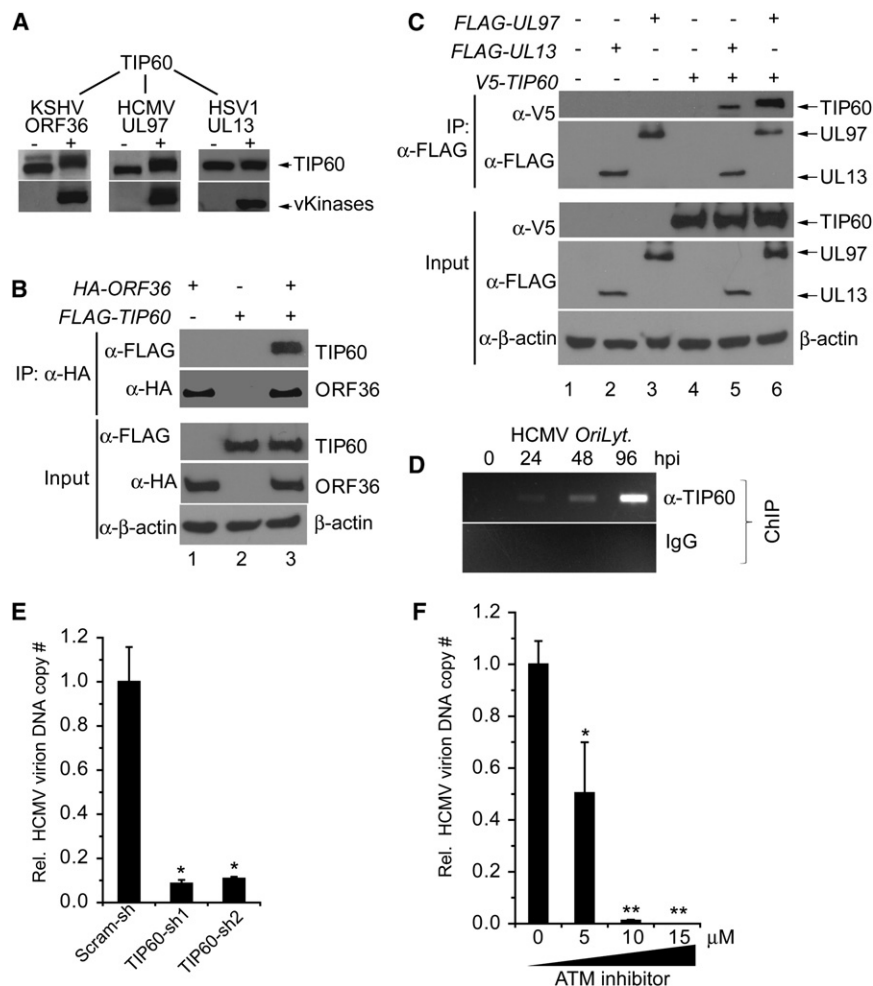


Figure 6. Conserved Role for TIP60 in Herpesvirus Replication

(A) Western blot analysis showing that KSHV ORF36 and HCMV UL97 increase the mobility of TIP60 in transfected HeLa cells.

(B and C) TIP60 coprecipitates with (B) KSHV ORF36, (C) HCMV UL97, and HSV1 UL13 using cotransfected 293T cells. Reciprocal immunoprecipitations are presented in Figures S6A and S6B. Input, 2% whole-cell lysate used for IP.

(D) TIP60 is recruited to HCMV lytic replication origin (OriLyt) at 24, 48, and 96 hr postinfection (hpi).

(E) TIP60 is required for efficient HCMV replication. Relative supernatant virion DNA from HCMV-infected HF cells (96 hpi) carrying control scramble shRNA (Scram-sh) or TIP60 shRNA (TIP60-sh) was determined with qPCR. The experiments were carried out in triplicate with \pm SD shown. * $p < 0.01$. See also Figure S6C.

(F) ATM inhibitor inhibits HCMV replication. Relative supernatant virion DNA from HCMV-infected HF cells (96 hpi) in the absence or presence of ATM inhibitor (KU55933) was determined with qPCR. The experiments were carried out in triplicate with \pm SD shown. * $p < 0.01$, ** $p < 0.005$. See also Figure S6D.

will assist in the design of assays for new and broadly effective antiherpesvirus therapeutics.

EXPERIMENTAL PROCEDURES

Kinase Assay

Phosphorylation of proteins on human protein arrays by herpesvirus protein kinases was assayed as previously described (Ptacek et al., 2005; Zhu et al., 2009). The list of the 4,191 unique proteins on this array can be found in Table S2 of Hu et al. (Hu et al., 2009). Detailed information is described in the Supplemental Experimental Procedures.

Immunoprecipitation and ChIP Assays

Cells were transfected using Lipofectamine 2000 (Invitrogen) or calcium phosphate, and the amount of DNA in each sample was equalized using vector DNA. Transfected cells were harvested 48 hr posttransfection, using RIPA lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% (v/v) NP40, 1% (w/v) deoxycholate 0.1% (w/v) SDS, and 1 mM EDTA) containing protease inhibitors and phosphatase cocktail I and II (Sigma) (Li et al., 2007). In Figure 3D, cells were treated with 20 μ M roscovitine for 12 hr before harvest to minimize the contribution of CDC2/CDK1. Immunoprecipitation and ChIP were carried out as described previously (Zhu et al., 2009). For phosphatase treatment, the immunoprecipitated complex was resuspended in 1 \times NEBuffer and incubated with 10 units of calf intestinal phosphatase (New England Biolabs) at 37°C for 1 hr. The complex was then eluted with Laemmli sample buffer and subsequently analyzed by SDS-PAGE and immunoblotting.

Histone Acetyltransferase Assay

TIP60 HAT activity was assayed using Flag-TIP60, Flag-TIP60S86/90A, and HAT dead Flag-TIP60 immunoprecipitated from 293T cells cotransfected with HA-BGLF4 or HA-BGLF4 kinase-dead mutant. Cells were treated with 20 μ M roscovitine for 12 hr before harvest, and TIP60 HAT activity was assayed using the HAT Assay Kit (Millipore) modified according to Sun et al. (2005).

We also demonstrate that TIP60 plays a positive role in transcriptional regulation of key lytic viral genes (Figure 7). BGLF4 has been implicated in facilitating viral egress from the nucleus by phosphorylating lamins (Lee et al., 2008). Interestingly, we find that TIP60 is recruited to the *LMP1* promoters after lytic induction and is needed for achieving normal levels of lytic *LMP1* transcription. *LMP1* downstream signaling is important for nuclear egress of virions (Ahsan et al., 2005), and our data suggest that TIP60-mediated activation of *LMP1* expression represents another mechanism by which BGLF4 promotes this aspect of infectious EBV production. TIP60's negative role in the establishment of latency and its positive role in lytic viral replication place TIP60 at the decision point between viral latency establishment and productive lytic replication (Figures 2 and 4D).

This work illustrates the value of high-throughput, unbiased approaches for the discovery of conserved viral targets. There are few drugs available to treat herpesvirus infections, and viral escape mutants develop upon extensive use of this limited repertoire. The herpesvirus protein kinases are attractive antiviral drug targets. However, developing broadly effective drugs requires knowledge of their common cellular substrates. The information provided by our common substrate identification

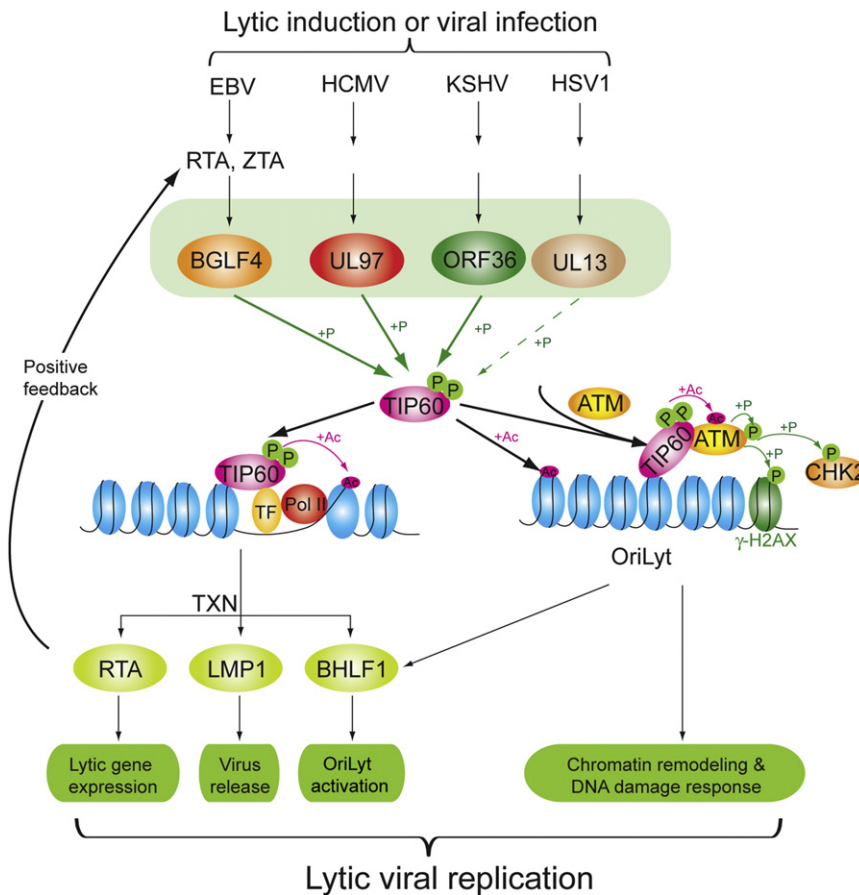


Figure 7. Model for Conserved Herpesvirus Kinases in Regulating Viral Replication through TIP60

The contribution of TIP60 activation by the conserved herpesvirus kinases to lytic replication, as illustrated mechanistically for EBV-infected cells. TXN, transcription; P, phosphorylation; Ac, acetylation; TF, transcription factor; Pol II, RNA polymerase II; OriLyt, lytic replication origin.

Virus Infection

For HCMV infection, HF cells were seeded into 24-well plates 1 day before infection. The cells were washed with PBS, and HCMV-luciferase virus (MOI = 1) was added to each well and incubated for 1.5 hr in 200 μ l serum-free Dulbecco's modified Eagle's medium (DMEM). Free viruses were removed with washing, and cells were incubated in medium containing 4% fetal bovine serum for 96 hr. To induce the EBV lytic cycle, Akata (EBV⁺) cells were treated with 50 μ g/ml of goat antihuman IgG (MP Biomedicals) for 24 hr, and SNU719 (EBV⁺) cells were treated for 24 hr with 20 nM of bortezomib (Fu et al., 2008).

Statistical Analysis

Statistical analyses employed a two-tailed Student's *t* test. A *p* value of ≤ 0.05 was considered statistically significant. The data are representative of at least two independent experiments, and values are given as the mean of replicate experiments \pm SD.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and two tables and can be found with this article online at doi:10.1016/j.chom.2011.08.013.

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J.Z., R.L., S.D.H., and H.Z. conceived the project. J.Z., R.L., S.D.H., and H.Z. designed experiments. S.D.T., R.F.A., and G.S.H. had input into experimental design. R.L. and J.Z. performed most of the experiments. Z.X., J.L., and J.Q. performed informatics and statistical analyses. S.H. and C.W. generated the human protein arrays. G.L. and M.-R.C. generated reagents. R.L., J.L., J.Z., P.D., and G.S.H. designed and performed HCMV infection assays. H.Z., R.L., J.Z., and S.D.H. wrote the manuscript.

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